Received: 11 January 2019 | Revised: 28 March 2019 | Accepted: 13 May 2019

DOI: 10.1111/ijlh.13070

ORIGINAL ARTICLE

ISLH International Journal of Laboratory Hematology WILEY

The applicability of multiparameter flow cytometry for the detection of minimal residual disease using different-from-normal panels to predict relapse in patients with acute myeloid leukemia after allogeneic transplantation

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Abstract

Background: The MRD status detected using multiparameter flow cytometry (MFC) before allogeneic hematopoietic stem cell transplantation (allo-HSCT) has crucial prognostic value for patients with acute myeloid leukemia (AML) in morphologic complete remission (CR). We designed a novel panel of antibodies to identify aberrant differentiation/maturation profiles of residual leukemia cells in patients who were not diagnosed at our institution, relapsed with an antigenic shift, or lack leukemia-associated immunophenotypes.

Methods: We compared the MRD status detected using MFC and real-time quantitative polymerase chain reaction (RT-qPCR) in the same 158 bone marrow samples collected from 44 AML patients carrying leukemia-specific fusion genes. The clinical performance of the MFC-based MRD status was analyzed in 168 AML patients who exhibited morphologic CR (135) or active disease (33) before HSCT.

Results: Strong concordance was found between MFC-based and RT-qPCR-based MRD status (κ = 0.868). Among the patients displaying CR, the positive MRD status detected using MFC was correlated with a worse prognosis [HRs (*P* values) for relapse, event-free survival, and overall survival: 4.83 (<0.001), 2.23 (0.003), and 1.79 (0.049), respectively]; the prognosis was similar to patients with an active disease before HSCT. Patients with a positive MRD before HSCT might experience a benefit from developing chronic graft-vs-host disease.

Conclusions: The assessment of MRD using our self-built different-from-normal AML-MRD detection panel exhibited reliable sensitivity, specificity, and accuracy. In addition, patients with a positive MRD status before transplantation may have higher risk of relapse and worse survival.

KEYWORDS

acute myeloid leukemia, allogeneic stem cell transplantation, minimal residual disease, multiparameter flow cytometry, relapse

Ziwei Wang, Mengqiao Guo and Yuesheng Zhang are contributed equally to this work.

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1 | INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is a curative treatment for acute myeloid leukemia (AML). However, leukemia relapse still accounts for most deaths after transplantation.[1-4] Based on accumulating evidence, a minimal residual disease (MRD) assessment has crucial prognostic value for patients with AML in morphologic complete remission (CR) after receiving allo-HSCT.[5-11] However, questions remain unanswered, particularly regarding the optimal method for MRD detection in patients with AML, which encompasses a wide range of myeloid neoplasms with diverse abnormalities. For some subtypes caused by or associated with a canonical genetic abnormality, quantitative polymerase chain reaction (PCR)-based MRD assessments have been proven reliable in detecting MRD.[12-14] However, its applicability is limited to approximately 40% of patients with AML who harbor suitable abnormalities.[5] Thus, multiparameter flow cytometry (MFC), which relies on the identification of aberrant immunophenotypes, may be a better choice for MRD assessments in patients with AML.

We designed a novel panel of antibodies to identify aberrant differentiation/maturation profiles and detect residual leukemia cells in patients with AML, including those who were not diagnosed at our institution, exhibited relapse with an antigenic shift, or lacked leukemia-associated immunophenotypes (LAIP). Because LAIPs are different-from-normal abnormalities in most cases,[5] our panel may effectively eliminate the difference between the two approaches with a reasonably high sensitivity and specificity. We evaluated the reliability and prognostic value of the MFC-based MRD in a retrospective study and found that MFC detection with our self-built panel was reliable for measuring residual disease in patients with AML. Moreover, patients with a positive MRD status before allogeneic transplantation had worse prognosis that was similar to patients with an active disease who underwent HSCT. Patients with a positive MRD before transplantation may not benefit from the myeloablative conditioning regimen (MAC) or the donor selection (eg, haploidentical transplantation), which require further verification in larger cohorts.

2 | PATIENTS AND METHODS

2.1 | Study cohort

One hundred sixty-eight patients with AML who received their first allo-HSCT at Changhai Hospital between September 2011 and July 2017 were included in this retrospective analysis, including 135 patients exhibiting morphologic CR and 33 patients with active disease before transplantation, with a median follow-up of 12.8 (1.8-82.7) months. The patients received stem cells from HLA-matched related or unrelated donors after undergoing a myeloablative conditioning regimen [cyclophosphamide plus total-body irradiation (Cy-TBI) regimen (Cy, 60 mg/kg/d, IV on days −5 to −4; TBI, 3 Gy twice daily on days −3 to −2), busulfan plus cyclophosphamide (BuCy) regimen (Bu, 0.8 mg/kg, IV q6h on days −8 to −5; Cy, 60 mg/kg/d, IV on days −4 to −3)] or a reduced-intensity conditioning regimen [FBA (fludarabine, 30 mg/m^2/d, IV for 2 hours on days −10 to −6; cytarabine, 1.5 g/m^2/d, IV on days −10 to −6 that started 4 hours after fludarabine and continued for 3 hours; Bu 0.8 mg/kg, IV q6h on days −5 to −3)]. If cells from an unrelated donor were used, the patient also received antithymocyte globulin (ATG) (Fresenius, Germany, 5 mg/kg/d, IV from days −4 to −1). The reduced-intensity conditioning regimen was only administered to patients exhibiting CR. Twenty-three patients received a haploidentical transplantation with a modified BuCy regimen as previously described.[15] All patients routinely underwent bone marrow aspiration and biopsy to evaluate the disease status before and after transplantation.

One hundred fifty-eight fresh bone marrow samples were collected at different time points form 44 patients with AML in morphologic CR who were diagnosed with leukemia-specific fusion genes (21 patients with AML1-ETO, 12 patients with CBFB-MYH11, and 11 patients with MLL fusion transcripts) to assess the clinical performance of MFC in detecting MRD. Tandem assessments of the residual disease status of these samples were performed using real-time quantitative polymerase chain reaction (RT-qPCR) and MFC.

The pre-HSCT MRD was detected using MFC in 135 patients in CR, and its prognostic value was then analyzed. The median follow-up of these patients was 13.0 (2.2-73.6) months.

2.2 | Detection of MRD

2.2.1 | MFC immunophenotyping

A five-color panel including cross-line CD markers and a four-color panel containing asynchronous CD markers were designed for AML-MRD detection. The CD markers used were CD34-PE/CD117-APC/CD45-PerCP/CD56-PE-Cy7/CD2-FITC/CD7-FITC (CD2 and CD7 were mixed in the same FITC channel) and CD34-APC/CD64-FITC/CD15-FITC/CD11c-PE/CD45-PerCP (CD64 and CD15 were mixed in the same FITC channel). The data were acquired and analyzed using a FACSAria II instrument with Diva software (BD Bioscience) or a Navios system with Kaluza software (Beckman Coulter). At least 300 000 events were collected. When identified, the abnormal cell population was quantified as a percentage of all nucleated cells. A cutoff value of 0.1% was accepted.

2.2.2 | RT-qPCR detection

The levels of leukemia-specific fusion genes, including AML1-ETO, CBFB-MYH11, and MLL fusion transcripts, were detected using RT-qPCR as previously described.[16] The results are reported as a transcript ratio calculated as (fusion gene/ABL1) × 100. The cutoff value was set to 0.001%.

2.3 | Statistical analysis

CR was defined as less than 5% blasts in the bone marrow and normalization of the peripheral blood count without circulating blasts.

Event-free survival (EFS) was defined as the time from transplantation to relapse or death resulting from any cause. Wilcoxon's rank-sum test was used to compare the continuous variables, and a chi-square test was used to compare the categorical variables. Intrarater reliability was estimated using a consistency test and is presented as the Kappa coefficient. The cumulative incidence of relapse was estimated using a competing risk analysis with nonrelapse mortality and was compared using the Gray test. Overall survival (OS) and EFS were calculated using the Kaplan-Meier method and compared using a log-rank test. Cox proportional hazard models were used to estimate the hazard ratios of the prognostic factors with a 95% confidence interval (CI). A P value ≤ 0.05 was considered statistically significant. R 3.4.4 software was used to analyze the data.

3 | RESULTS

3.1 | Analysis strategies for MFC-based MRD

Many patients with leukemia diagnosed in other hospitals of China visit our department for HSCT. Many of them, however, were unable to provide their primary leukemia immunophenotypes. We designed 5- and 4-color different-from-normal panels (DFN) containing both cross-line and asynchronous CD markers to quickly and easily track residual leukemia cells in these patients and other patients displaying shifting immunophenotypes during treatment. In the 5-color panel, the gated CD34-positive cells were further precisely focused on CD45/SSC to remove some nonspecific backgrounds because CD45 is always expressed at low levels on the leukemia blasts. The $CD34^+CD2/7^+$ and $CD34^+CD56^+$ cells represent the abnormal blasts if their quantitative value exceeds the 0.1% cutoff value (Figure S1A). $CD117^+CD2/7^+$ and $CD117^+CD56^+$ cells in the same test tube are also considered residual leukemia cells using the same cutoff value (Figure S1B). When cells displaying asynchronous expression ($CD34^+CD11c^+$ and $CD34^+CD64/15^+$) exceeding the cutoff values (0.1%) were identified in the 4-color panels, positive MRD results were also established (Figure S1C). The largest quantitative value of these gated abnormal cells was reported as the final MRD value. In those patients with higher values for residual leukemia cells (always greater than 1%), the analysis strategies might vary (Figure S2). The abnormal cells always gathered or were gated in the CD45/SSC image. In these cases, the expression of the other detected CD markers was displayed in the two-by-two combined scatter plots to determine their LAIP, and the total proportion of

TABLE 1 Characteristics of 135 CR AML patients

Parameters	Study cohort (N = 135)	Pre-HSCT MRD^{pos} (N = 49)	Pre-HSCT MRD^{neg} (N = 86)	P value
Age at transplantation, years (range)	40 (11-60)	36 (16-58)	41 (11-60)	0.084
Gender, N (%)				
Male	73 (54.07)	29 (59.18)	44 (51.16)	0.369
Female	62 (45.93)	20 (40.82)	42 (48.84)	
ELN risk classification				
Favorable	11 (8.15)	5 (10.20)	6 (6.98)	0.079
Intermediate	107 (79.26)	34 (69.39)	73 (84.88)	
Adverse	17 (12.59)	10 (20.41)	7 (8.14)	
Disease status at transplantation,				
N (%)				0.027
First CR	112 (82.96)	36 (73.47)	76 (88.37)	
CR2/3	23 (17.04)	13 (26.53)	10 (11.63)	
Donor type, N (%)				
Matched related	71 (52.59)	25 (51.02)	46 (53.49)	0.468
Unrelated	48 (35.56)	16 (32.65)	32 (37.21)	
Haploidentical	16 (11.85)	8 (16.33)	8 (9.30)	
Donor-patient sex matching				
Female to male	24 (17.78)	10 (20.41)	14 (16.28)	0.546
Others	111 (82.22)	39 (79.59)	72 (83.72)	
Conditioning regimen, N (%)				
Myeloablative	84 (62.22)	35 (71.43)	49 (56.98)	0.096
Reduced intensity	51 (37.78)	14 (28.57)	37 (43.02)	

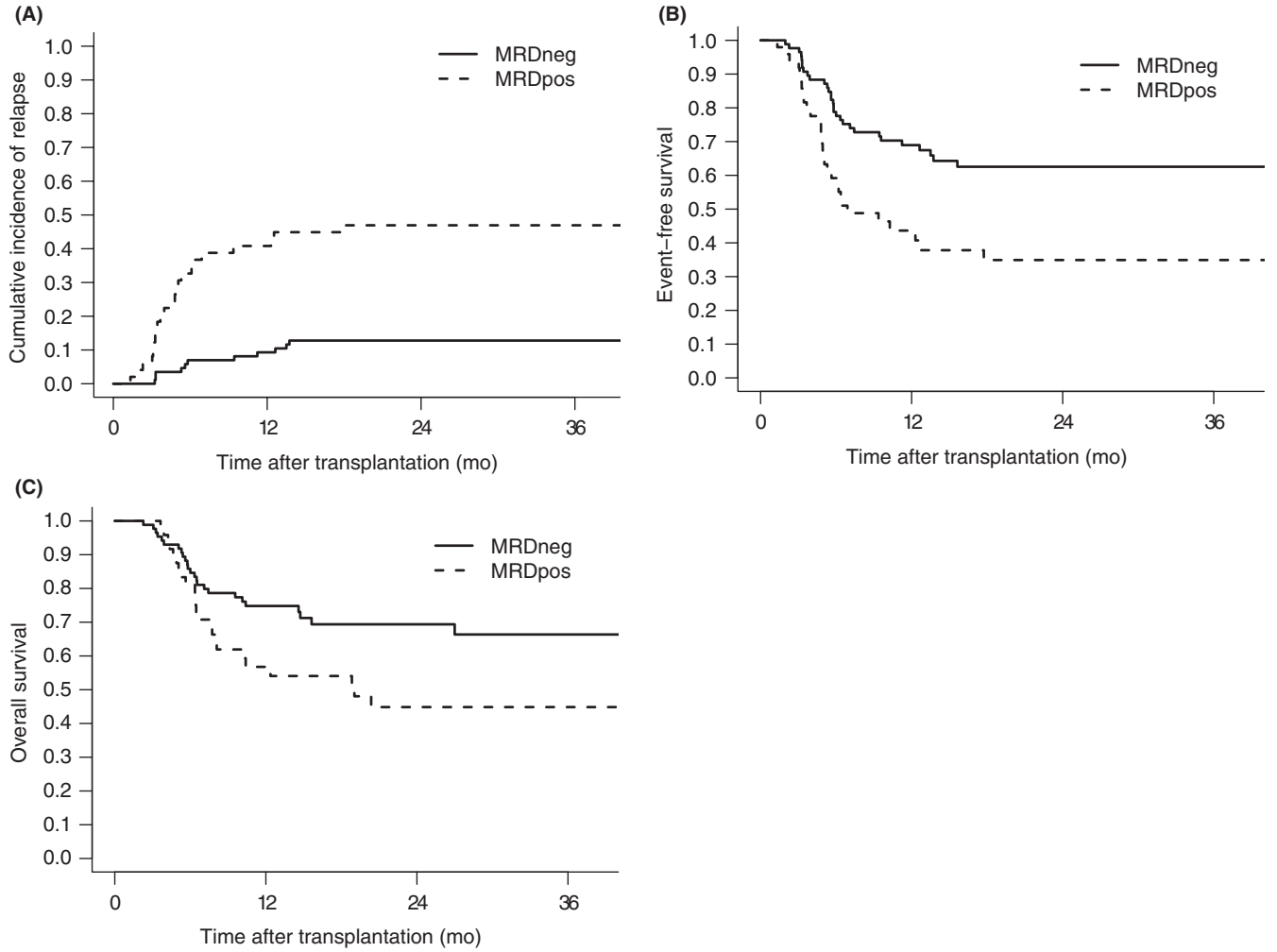

FIGURE 1 Cumulative rates of (A) relapse ($P < 0.001$), (B) EFS ($P = 0.001$), and (C) OS ($P = 0.026$) in the pre-HSCT MRD^{pos} (N = 49) and MRD^{neg} groups (N = 86)

the abnormal cells gated on CD45/SSC, CD34/SSC, or CD117/SSC was reported as the final MRD value (Figure S2).

3.2 | Reliability of MRD detected using MFC

We collected 158 bone marrow samples from 44 patients in morphologic CR who were diagnosed with leukemia-specific fusion genes, detected the residual disease status of the same sample using MFC and RT-qPCR, and estimated the concordance to confirm the reliability of our flow cytometry panel in detecting MRD among patients with AML. Thirty-seven samples (23.42%) were positive using both MFC and RT-qPCR, whereas 113 samples (71.52%) were negative using both methods. Three samples (1.90%) were negative using MFC but positive using RT-qPCR, and 5 samples (3.16%) were positive using MFC but negative using RT-qPCR (Table S1). Strong agreement existed between the two methods, with a Kappa coefficient of 0.868 (95% CI: 0.780-0.957). The sensitivity, specificity, and accuracy of the MRD detected using MFC were 88.10%, 97.41%, and 94.9%, respectively.

3.3 | Associations between pre-HSCT MRD detected using MFC and outcomes among patients with AML

One hundred thirty-five patients with AML in CR were enrolled in this analysis, and the 2-year OS, EFS, and cumulative incidence of relapse were 60.4% (95% CI: 51.3%-69.5%), 52.5% (95% CI: 43.5%-61.6%), and 25.2% (95% CI: 17.8%-32.5%), respectively. Among these patients, 49 had an MRD-positive status (MRD^{pos}), and 86 had an MRD-negative status (MRD^{neg}) before transplantation. Compared with the MRD^{neg} group, more patients had a late disease status [second or third complete remission (CR2/3)] in the MRD^{pos} group. The details of these patients are summarized in Table 1. Twenty-three MRD^{pos} patients and 11 MRD^{neg} patients progressed to morphologic relapse by the day of the data cut-off. The 2-year cumulative incidence of relapse was 46.9% (95% CI: 33.0%-60.8%) among MRD^{pos} patients and 12.8% (95% CI: 6.6%-19.0%) among MRD^{neg} patients ($P < 0.001$, Figure 1A). The MRD^{pos} patients also had worse EFS [34.9% (95% CI: 20.1%-49.7%) vs 62.5% (95% CI: 51.6%-73.5%); $P = 0.001$ (Figure 1B)] and OS

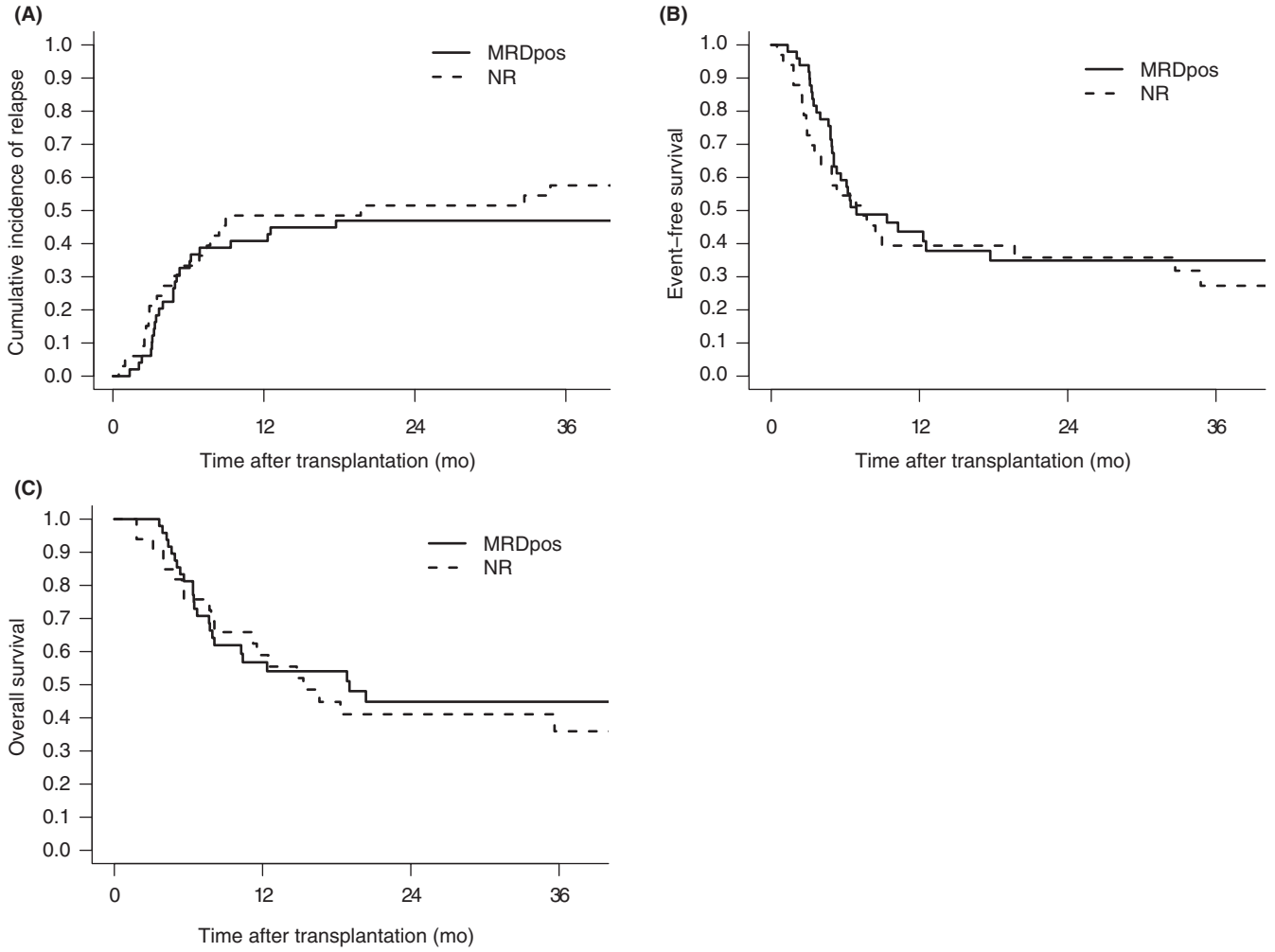

FIGURE 2 Cumulative rates of (A) relapse ($P = 0.399$), (B) EFS ($P = 0.527$), and (C) OS ($P = 0.686$) in the pre-HSCT^{pos} group (N = 49) and active disease group (N = 33)

[44.9% (95% CI: 29.0%-60.8%) vs 69.4% (95% CI: 58.8%-80.0%); $P = 0.026$ (Figure 1C)].

We also compared the prognosis of 107 patients with AML in the intermediate-risk group, including 73 MRD^{pos} patients and 34 MRD^{neg} patients. Similar to patients with all kinds of stratification, patients with AML in intermediate-risk group with a positive MRD status also had higher relapse rate and worse survival than MRD^{neg} patients [the 2-year cumulative incidences of relapse were 54.54% (37.07%-72.02%) and 11.00% (3.72%-18.20%), respectively ($P < 0.001$); the 2-year EFS rates were 32.63% (15.90%-49.37%) and 64.16% (52.28%-76.03%), respectively ($P = 0.001$); and the 2-year OS rates were 41.25% (22.79%-59.72%) and 72.31% (61.43%-83.18%), respectively ($P = 0.012$)].

3.4 | Comparison of the prognosis between patients exhibiting CR with a positive MRD status and patients with active disease

Another 33 patients with active disease (relapsed or nonremission) before HSCT were enrolled to compare the prognosis with MRD^{pos} patients. The characteristics of these patients are summarized in Table S2. The 2-year cumulative incidence of relapse was 51.5% (95% CI: 34.0%-69.0%) among the patients with active disease and 46.9% (95% CI: 33.0%-60.8%) among the MRD^{pos}-CR patients ($P = 0.399$) (Figure 2A). The EFS and OS at 2 years were not significantly different between the two groups [EFS 35.8% (95% CI: 18.7%-52.9%) for patients with active disease and 34.9% (95% CI: 20.1%-49.7%) for MRD^{pos}-CR patients ($P = 0.527$) (Figure 2B); OS 41.1% (95% CI: 22.5%-59.7%) for patients with active disease and 44.9% (95% CI 29.0%-60.8%) for MRD^{pos}-CR patients ($P = 0.686$) (Figure 2C)].

3.5 | Pre-HSCT MRD status as an independent prognostic factor for morphological relapse

A multivariate analysis of the OS, EFS, and relapse rates was performed in the 135 patients exhibiting CR to analyze the predictive value of the pre-HSCT MRD status for post-transplantation outcomes. These models indicated that a pretransplantation MRD-positive status was an independent risk factor for OS, EFS, and the cumulative incidence of relapse (Table 2). Furthermore, the development of chronic graft-versus-host disease (cGVHD) was associated with a decreased risk of relapse.

TABLE 2 Multivariate analysis for OS, EFS, and the cumulative incidence of relapse

Outcomes	Variables	HR (95% CI)	*P* value
	MRD status before transplantation		0.049
	Negative	1	
	Positive	1.79 (1.01-3.19)	
	Chronic GVHD		<0.001
OS	No	1	
	Yes	0.26 (0.13-0.55)	
	Acute GVHD		
	No/Grade I	1	
	Grade II	1.21 (0.37-3.99)	0.750
	Grades III-IV	14.11 (6.27-31.75)	<0.001
	MRD status before transplantation		0.003
	Negative	1	
	Positive	2.23 (1.32-3.75)	<0.001
	Chronic GVHD		
EFS	No	1	
	Yes	0.34 (0.18-0.63)	
	Acute GVHD		
	No/Grade I	1	0.885
	Grade II	0.92 (0.28-2.98)	<0.001
	Grades III-IV	4.43 (2.23-8.83)	
Relapse	MRD status before transplantation		<0.001
	Negative	1	
	Positive	4.83 (2.33-10.02)	
	Chronic GVHD		0.019
	No	1	
	Yes	0.41 (0.19-0.86)	

TABLE 3 Multivariate analysis for prognosis in patients with positive MRD status

Outcomes	Variables	HR (95% CI)	*P* value
OS	Chronic GVHD		0.007
	No	1	
	Yes	0.18 (0.05-0.64)	
	Acute GVHD		
	No/Grade I	1	
	Grade II	1.10 (0.25-4.82)	0.896
	Grades III-IV	8.82 (2.23-34.85)	0.002
EFS	Chronic GVHD		0.001
	No	1	
	Yes	0.35 (0.19-0.64)	
	Acute GVHD		
	No/Grade I	1	
	Grade II	0.78 (0.24-2.51)	0.674
	Grades III-IV	4.60 (2.34-9.06)	<0.001
Relapse	Chronic GVHD		0.031
	No	1	
	Yes	0.35 (0.14-0.91)	

Another multivariate analysis (including factors such as the ELN classification, donor-patient sex matching, donor type, conditioning regimen, ATG, aGVHD, and cGVHD) of the 49 MRD^{pos} patients was performed, revealing that these patients may not benefit from the haploidentical donor selection or the stronger conditioning regimen (Table 3). Only the benefit of cGVHD after transplantation was significant in the current analysis.

4 | DISCUSSION

Allogeneic stem cell transplantation is the most preferred post-remission therapy for patients with AML.[17] However, approximately 30%-40% of patients with AML experience relapse after allo-HSCT, and their outcomes are dismal.[1,2] Thus, refining the risk stratification that could be used to identify patients at a higher risk of relapse is crucial for reducing relapse and improving prognosis. The detectable MRD in patients exhibiting morphologic CR predicts an increased risk of leukemia recurrence and worse outcomes.[3,5,7,18,19] However, currently, a uniform approach for the detection of MRD is unavailable because AML is genetically diverse. PCR-based MRD detection of mutant transcripts is the most sensitive approach and has been widely used to monitor MRD in patients with chronic myelogenous leukemia (CML), Philadelphia chromosome-positive acute lymphoblastic leukemia (Ph + ALL), acute promyelocytic leukemia (APL),[20] and a small group of AML subtypes (eg, NPM1/RUNX1-RUNX1T1/CBFB-MYH11/PML-RARA-positive samples).[5] However, the lower applicability limits its usage. Multigene next-generation sequencing (NGS) is another potential molecular method for MRD detection that is applicable for most patients with AML. However, the consensus list of genes and mutations used in NGS technology requires further investigation, particularly numbers of somatic mutations, which may not represent residual leukemic cells. As a result, MFC-based MRD assessments may be preferable due to several advantages, such as wide applicability, rapid and accurate quantification of leukemia cells and relative sensitivity. The following two approaches are used to identify residual leukemic cells in patients with AML using MFC: LAIPs and different-from-normal. Currently, LAIPs are widely used and capture approximately 90% of patients.[21,22] However, this method requires knowledge of the immunophenotypes of primary leukemia cells in each individual. Personalized LAIP panels also need more antibody reserves with different fluorescent labels

and a greater laboratory workload. However, LAIP information is lacking for many patients, partially because of increases in patient mobility in China, and relapsed leukemic cells carrying an antigenic shift decrease the efficacy of LAIP methods. We designed different-from-normal panels containing antibodies to identify aberrant differentiation/maturation profiles to track residual leukemia cells in these patients. We compared the MRD status of 158 bone marrow samples collected from 44 patients who were diagnosed with leukemia-specific fusion genes and observed a strong concordance between MFC-based and RT-qPCR-based MRD detection, indicating that MFC-based MRD detection using our panel exhibited a reliable sensitivity and specificity. Disconcordance may be due to the relative quantification of abnormal transcripts using RT-qPCR and the accurate quantification of leukemic blast cells using MFC. Because LAIPs are different-from-normal in most cases, our new panel may eliminate the differences between the two approaches with reasonably high sensitivity and specificity.

We analyzed the relationship between the MRD status before allo-HSCT and prognosis of 135 patients with AML in CR to further evaluate the clinical performance of MFC-based MRD detection. As shown above, a pretransplantation MRD^{pos} status correlated with an increased risk of relapse and worse survival, consistent with most other studies.[7,8,19] Meanwhile, patients with a positive MRD status before HSCT may not benefit from haploidentical transplantation or myeloablative conditioning regimen. However, Liu et al did not observe an association between the pretransplantation MRD status and the outcomes of patients with AML receiving haploidentical transplants.[23] This discrepancy may be mainly due to the limited number of patients in the current analysis. Furthermore, a routine prophylactic donor lymphocyte infusion was administered to patients with refractory disease or in the second remission in that study, which may be another explanation for the discrepancy.

In summary, the continuous improvements in multiparameter flow technology and reagents have increased the accuracy and applicability of the MFC-based MRD detection in patients with AML, offering further valuable therapeutic strategies, and the assessment of the MRD status using our self-built different-from-normal AML-MRD detection panel displayed reliable sensitivity, specificity, and accuracy. In addition, patients with a positive MRD status before HSCT may have a higher risk of relapse and worse survival. Based on these data, pre-emptive therapy before transplantation is important for patients with a positive MRD status, and the reduced use of immunosuppressive medications along with donor lymphocyte infusion may be beneficial for these patients. However, the increased risk of transplant-related mortality should be carefully considered. This retrospective analysis had some limitations, such as the relatively small sample size and the variation in the donor type and conditioning regimens; thus, well-designed prospective studies involving larger sample sizes are needed in the future.

CONFLICT OF INTEREST

The authors have no competing interests to declare.

AUTHORS' CONTRIBUTIONS

ZWW collected and verified patients' information, analyzed the data, and wrote the manuscript. MQG and YSZ collected and verified patients' information. GST designed the study, and wrote and modified the manuscript. YSZ, GST, and other authors involved in the treatment of the patients or MRD monitoring by MFC or RT-PCR.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

How to cite this article: Wang Z, Guo M, Zhang Y, et al. The applicability of multiparameter flow cytometry for the detection of minimal residual disease using different-from-normal panels to predict relapse in patients with acute myeloid leukemia after allogeneic transplantation. *Int J Lab Hematol*. 2019;00:1–8. https://doi.org/10.1111/ijlh.13070